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Regulation of the Glutathione S-Transferase P1 Expression in Melanoma Cells

Andrii Slonchak¹, Agata Chwieduk²,
Joanna Rzeszowska-Wolny³ and Maria Obolenskaya¹

¹*Institute of Molecular Biology and Genetics National Academy of Sciences of Ukraine*

²*Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology*

³*Silesian University of Technology*

¹*Ukraine*

^{2,3}*Poland*

1. Introduction

Melanoma is a malignant tumor that results from DNA damage and related mutations in melanocytes (Rass & Reichrath, 2008). It usually occurs in result of collision of ultraviolet (UV) photon with a chromophore in a skin cell, production of reactive oxygen species which attack the melanocytes and induce oxidative stress with the DNA damage by oxidation. Conceptually, oxidative stress results from a change in the equilibrium between production of pro-oxidant and their consumption or deactivation, favoring an excess of pro-oxidant that have noxious consequences at the molecular and cellular levels (Ananthaswamy & Pierceall, 1990). There are several mechanisms and barriers that protect the body against ultraviolet radiation: the stratum corneum, the outermost layer of the skin, and melanin pigment attenuate penetration of UV into the skin; antioxidative enzymes detoxify and metabolize reactive oxygen species; DNA repair systems protect cells from UV-induced lesions (Hoeijmakers, 2001). The cellular antioxidant defense relies to the great extent on a powerful sulfur redox chemistry response in which glutathione S-transferases are active players.

Glutathione S-transferases (GSTs) comprise a multigene superfamily of enzymes that catalyze the conjugation of electrophilic toxic compounds with glutathione, playing a key role in phase II of detoxification (Hayes et al., 2005). GSTs are dimeric enzymes with subunit polypeptides organized into several cytosolic families (α , μ , π , σ , θ , ζ , ω) and one microsomal form (κ) (Frova, 2006). The human Pi class isoform of GSTs (GSTP1-1) is widely expressed in epithelial tissues and is the dominant isoform in lung, brain, skin, esophagus, erythrocytes and also in fetal tissues including liver, lung, kidney, and placenta (Moscow et al., 1989). This isoform has been proposed to be a caretaker gene, protecting cells against genome damage mediated by oxidants and electrophiles from inflammation or environmental exposure. Besides its typical role in detoxification of electrophilic toxic compounds this enzyme carries out a wide range of other functions, such as removal of reactive oxygen species and generation of S-thiolated proteins during the oxidative stress (Hayes & Strange, 1995), binding and transfer of different ligands (Oakley et al., 1999), modulation of signaling pathways (Adler et al., 1999; Villafania et al., 2000; Wu et al., 2006; Zhao et al., 2006),

conjugation and transport of steroid hormones, dinitrosyl-diglutathionyl-iron complex storage and metabolism (De et al., 2003; Pedersen et al., 2007; Ricci et al., 2003; Turella et al., 2003).

The GSTP, GSTT and GSTM (GSTs) isoforms prevent carcinogenesis through inactivation of reactive electrophiles by conjugation to reduced glutathione. The human GSTP1 isoform also possesses selenium-independent peroxidase activity and restores organic hydro- and endoperoxides thus additionally protecting DNA from oxidative damage (Tan et al., 1988).

Therefore, it is biologically plausible that a deficiency in the activity of GSTs may contribute to the risk of UV-induced melanoma. There are many evidences that mutation-associated reduction of GST activity predisposes to melanoma (Bu et al., 2007; Dolzan et al., 2006). However, mutations are not the single cause of GSTP1 insufficiency. This phenomenon occurs in different tumors as a result of *GSTP1* gene repression by methylation of promoter or via specific set of transcription factors (Henderson et al., 1998).

Unlike down-regulation of *GSTP1* expression its overexpression is associated with the development of multidrug resistance (Harbottle et al., 2001). It is known that melanoma is the most aggressive form of skin cancer, which is notoriously resistant to all current modalities of anticancer therapy, including chemotherapy and γ -irradiation. Numerous genetic, functional and biochemical studies suggest that melanoma cells become insensitive to chemotherapeutic drugs by exploiting their resistance to apoptosis and by reprogramming their signaling pathways (Helmbach et al., 2001). It was demonstrated that *GSTP1* and multidrug resistance protein 1 (*MRP1*) are overexpressed in multi-drug resistant melanoma cells and responsible for resistance to chemotherapy (Depeille et al., 2005).

Thus, reactive oxygen species that damage melanocytes DNA, highly contribute to neoplastic transformation of melanocytes, what leads to the assumption that melanocytes should have reduced protection from electrophiles particularly due to reduced activity of corresponding detoxification enzymes. However, malignant melanoma is highly resistant to chemotherapy and γ -irradiation that points to the elevated detoxification and antioxidant activity in the tumor cells. This contradiction allowed us to suppose that activity of detoxification system, and particularly of *GSTP1*, may be down-regulated during neoplastic transformation, making the melanocytes sensitive to reactive oxygen species generated by UV-irradiation, and up-regulated by the therapeutic agents, conferring protection against them. To verify this assumption we decided to study the *GSTP1* expression and its regulation at transcriptional level in human malignant melanoma cell line Me45 before and after γ -irradiation. Due to the important role of *GSTP1* in health and disease the regulation of *GSTP1* gene expression is in the focus of interests of numerous researchers and clinicians, but according to our knowledge, the potential role of *GSTP1* regulation in the etiology and the progress of melanoma were not studied before. Thereby we have addressed the following questions:

- What is the level of *GSTP1* expression in melanoma cells in comparison with the other malignant cell types and how does it change after γ -irradiation;
- Which cis- and trans-elements (transcription factors) participate in regulation of *GSTP1* transcription in melanoma cells;
- Which transcription factors may contribute to induction of this gene in response to γ -irradiation.

GSTP1 gene expression is regulated at transcriptional, post-transcriptional, translational and post-translational levels (Daniel, 1993; Jhaveri et al., 1997; Moffat et al., 1997). We focused our attention on the regulation at transcriptional level particularly by transcription factors

and cis-elements of the 5'-flanking region of the gene. Information about the structure of *GSTP1* promoter and transcription factors that were reported to interact with specific sites is summarized in Fig.1.

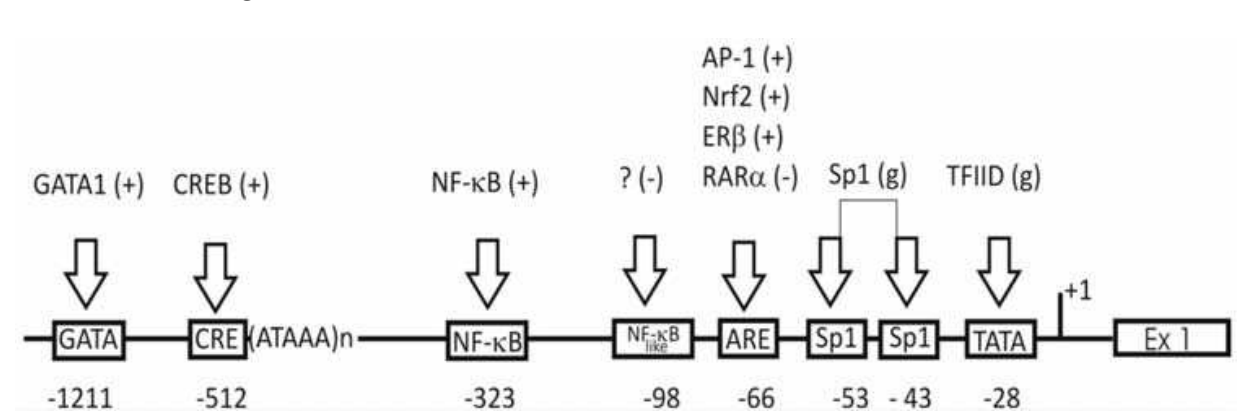


Fig. 1. Structure of the human *GSTP1* gene 5'-regulatory region and transcription factors known to interact with the promoter elements (Slonchak et al., 2009): (+) – positive regulation, (-) – negative regulation, (g) – general transcription factors

The region spanning from - 80 to - 8 contains a TATA-box at - 28 to - 24, two Sp1 binding sites (G/C-boxes) located at - 43 to - 38 and - 53 to - 48 and antioxidant response element (ARE) at - 59 to - 66 (Cowell et al., 1988) and is essential for initiation of *GSTP1* transcription. Binding of Sp1 to the proximal G/C-box (Moffat et al., 1996a) and a transcription factor from AP-1 (Morrow et al., 1990) or NF-E2 family (Nishinaka et al., 2007) to ARE located in this minimal promoter are required to initiate the transcription. ARE is also responsible for gene induction by retinoic acid (Xia et al., 1996), estrogens (Montano et al., 2004), phorbol esters (Duvoix et al., 2004b), curcumin (Nishinaka et al., 2007) and doxorubicin (Duvoix et al., 2004a). The region spanning from - 1212 to - 90, which contains NF-κB-like element at - 98 to - 90 (Moffat et al., 1996b), NF-κB binding site at - 323 to - 314 (Morceau et al., 2004), cAMP response element (CRE) at - 512 to - 505 (Lo & li-Osman, 2002) and GATA-1 binding site at - 1212 to - 1207 (Morceau et al., 2000) is not essential for initiation of *GSTP1* transcription but mediates gene's responsibility to different stimuli. The NF-κB-like element mediates up-regulation of the *GSTP1* gene transcription in response to oxidative stress by binding NF-κB (Xia et al., 1996) and transcription silencing in MCF7 breast cancer cells (Moffat et al., 1996b). NF-κB binding site was reported to bind NF-κB and stimulate gene expression in response to TNFα treatment in leukemia cells (Morceau et al., 2004). CRE site binds CREB and mediates cAMP-dependent gene activation in lung cancer cells (Lo & li-Osman, 2002) and GATA-1 binds GATA-1 transcription factor and mediates the induction of *GSTP1* transcription during hemin-induced differentiation of K562 cells (Schnekenburger et al., 2003).

Despite the vast literature devoted to *GSTP1* enzyme the functional characteristics of response elements in gene promoter and tissue-specific regulation of *GSTP1* transcription are poorly understood and were not studied in melanoma cells. To fill this gap we performed the functional analysis of *GSTP1* promoter in the human melanoma cell line. We examined *GSTP1* expression level in these cells, the functional role of cis-elements in *GSTP1* promoter and identified transcription factors binding with these elements. We also conducted the experiments to analyze the ability of *GSTP1* gene to be induced by γ-irradiation.

In this research we used cell line Me45, which is a human malignant melanoma cell line derived from a metastasis of skin melanoma into lymph node at 35 years old male. It was established in 1997 in the Department of Radiobiology of M. Sklodowska-Curie Cancer Centre and Institute of Oncology in Gliwice. Identity of melanoma cells was confirmed by immunocytochemical reaction with monoclonal antibodies to HMB50, S-100 and Melan A (Kumala et al., 2003; Przybyszewski et al., 2004; Rzeszowska-Wolny et al., 2009).

2. Materials and methods

2.1 Cell culture

Human melanoma cells Me45, mammary carcinoma cells MCF7, epithelial breast cells Hbl-100, bronchoalveolar carcinoma cells H358, alveolar carcinoma cells A549, erythroleukaemia cells K562, hepatocellular carcinoma cells HepG2, lung cancer cells Hct116, immortalized human bronchial epithelial cell BEAS2B, adrenal carcinoma cells FN-H296, mammary carcinoma cells MCF10A and choriocarcinoma cells BeWo were propagated in Dulbecco's modified Eagle's medium nutrient mixture F12 HAM (Sigma, USA) containing 12% fetal bovine serum (Gibco, USA) and 100 µg/ml gentamicine in tissue culture flasks. Culture medium for choriocarcinoma cells BeWo was also supplemented with MEM Non-Essential Amino Acid Solution 1x (MEM NEAA 100x, Gibco, USA). Incubation was performed at 37 °C in humidified atmosphere of air containing 5% CO₂.

2.2 γ-Irradiation of the cells

Me45 and BeWo cells grown to 80% confluence in 3 cm culture dishes were γ-irradiated at 1 Gy/min from a Clinac 600 GMV (Varian, Palo Alto, CA, USA) at room temperature to a total dose of 2 Gy corresponding to therapeutic dose and then incubated at 37 °C during 0.25-12 h. After incubation RNA was extracted from cells using RNA Mini kit (A&A Biotechnology, Poland).

2.3 Estimation of *GSTP1* expression

2.3.1 RNA isolation and reverse transcription

Total RNA was isolated from cultured cells grown to 80% confluence in 6-well plates using Total RNA Mini kit (A&A Biotechnology, Poland). RNA (1 µg/lane) was analyzed by electrophoresis in denaturing agarose gel. First strand cDNA was synthesized from 1 µg of total RNA using iScriptcDNA Synthesis kit (Bio-Rad, USA). The 20 µl reaction contained 4 µl 5x iScript Reaction Mix, 1 µl iScriptReverse Transcriptase and 2 µg of total RNA. The reaction mixture was incubated 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. The resulted mixture was used directly for amplification by real-time PCR.

2.3.2 Quantitative real-time PCR

GSTP1 gene expression was determined by real-time PCR on DNA Engine Peltier Thermal Cycler with Chromo4 Real-Time PCR Detector (Bio-Rad, USA). PCR was performed in 20 µl reaction mixture contained 2 µl cDNA, 10 µl 2x Real Time PCR Master Mix SYBR Set A (A&A Biotechnology, Poland) and 10 pmole of each primer - *GSTP1L*: 5'-CCCAAGTTCCAGGACGGAGA-3' and *GSTP1R*: 5'-GCCCCGCCTCATAGTTGGTGT-3'. Samples were denatured at 94 °C for 4 min, and cDNA products were amplified with 45 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for

30 s. The specificity of amplification was checked using post-PCR melting curves analysis. During exponential phase of PCR reaction, the crossing threshold (C_T) was determined for each amplification curve. MCF10A cell line was used as calibrator. Results were normalized to RPL41 ribosomal protein mRNA quantified with qRT-PCR performed under the same conditions. The relative quantification ratio was determined based on C_T method with an efficiency correction using Opticon Monitor 3 Software (Bio-Rad, USA).

2.3.3 Semi-quantitative western blot analysis

Cells grown to 80% confluence in 6-well plates were lysed in 500 μ l of RIPA Buffer (Thermo Scientific, USA) supplemented with Complete Proteinase Inhibitor Cocktail (Roche, Switzerland). Protein concentration was determined using Bradford reagent (Bio-Rad, USA). Total proteins (25 μ g) of each cell lysate were resolved in 12% SDS-PAGE according to Laemmli method (Laemmli, 1970), and electroblotted onto Immobilon-P Transfer Membrane (Millipore, USA). Immunodetection of GSTP1 protein was accomplished with rabbit polyclonal anti-GSTP1 antibody (StressGen, USA) diluted 1:1000 with 3% BSA in TBS following the procedure recommended by antibodies manufacturer. To detect the immunoreactive proteins, horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA) diluted 1:10000 with 3% BSA in TBS, and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) was used. Visualization of the immunoreactive bands was achieved by exposing the membrane to the X-ray film. Films were developed and scanned. Band intensities were quantified with image analysis software GelPro 4.5 (Media cybernetics, USA). The amount of protein was normalized by Ponceau S staining of the membranes after electrotransfer.

2.4 Analysis of functional activity of cis-elements in *GSTP1* promoter and identification of transcription factors interacting with them

2.4.1 Reporter constructs and transient transfection assay

Fragments of *GSTP1* gene promoter were prepared by PCR. The oligonucleotide 5'-ACTCACTGGTGGCGAAGACT-3' (position +15 to +35) was used as the downstream primer for all constructions. Each of the following oligonucleotides was used as the upstream primers to amplify promoter fragments: 5'-CAT AAA CAC CAA CCT CTT CCC C-3' (position -1379 to -1357) for pGSTP1415, 5'-ATA GCC TAA GGC ACA GCC AC-3' (position -1162 to -1142) for pGSTP1197, 5'-TTT CCT TTC CTC TAA GCG GC-3' (position -405 to -385) for pGSTP440, 5'-AGT CCG CGG GAC CCT CCA GA-3' (position -105 to -85) for pGSTP140 and 5'-AGA GCG GCC GGC GCC GTG AC-3' (position -85 to -64) for pGSTP120. Primers were designed by Vector NTI Advance 10.0 software (Invitrogen, USA) using *GSTP1* gene sequence AY324387. PCR was performed in 25 μ l of reaction mixture containing 1x QIAGEN PCR Buffer, 1.5 mM $MgCl_2$, 200 μ M each dNTP, 10 pmoles of each primer, 1x Q-solution, 500ng DNA and 2.5u Taq-DNA polymerase. DNA was denatured at 94 °C for 10 min, and promoter fragments were amplified with 30 cycles - denaturation at 94 °C for 30 s, annealing and extension at 60 °C for 120 s (for 1415bp fragment), 90 s (for 1197 bp fragment), 60 s (for 440 bp fragment) and 30 s (for 140 and 120 bp fragments). Final extension step was performed at 72 °C for 15 min. The amplified products were gel-purified using Gel-Out Kit (A&A Biotechnology, Poland) and subcloned into pCR2.1®-TOPO® vector (Invitrogen, USA), excised by KpnI and XhoI and religated into KpnI and XhoI linearized and dephosphorylated pGL3-basic plasmid (Promega, USA). DNA template for

PCR was isolated from human peripheral blood using Genomic Mini Kit (A&A Biotechnology, Poland). All PCR reagents and kits for plasmid isolation were obtained from Qiagen Inc. (USA). Plasmids for transfection were isolated using EndoFree Plasmid Maxi Kit (Qiagen Inc., USA). All enzymes were from MBI Fermentas (Lithuania). Sequences of relevant regions of the reporter constructs were confirmed by sequencing in both directions in Oligo.pl DNA IBB PAN Service (Poland).

Me45 cells were grown in 24-well plates to 60% confluence and transfected with 500 ng of pGSTP together with 25 ng of pRL-TK plasmid (Promega, USA) per well using Lipofectamine™ LTX and PLUS™ reagents (Invitrogen, USA). After 20h the culture medium was removed, cells were washed with PBS and lysed in 250 µl of Passive lysis buffer (Promega, USA). Firefly and *renilla* luciferase activities were assessed in 5 µl of the lysates using Dual Luciferase® Reporter Assay System (Promega, USA). Light emission resulting from luciferase activity was measured in Lumat LB 9506 luminometer (Berthold technologies, USA) by integration of peak light emission during 10 s at 25 °C. The ratio between arbitrary firefly and *renilla* luciferase light units was calculated for each probe. Each experiment was repeated three times with triplications in each one.

2.4.2 Electrophoretic mobility shift assay

Me45 cells were grown in T225 flasks to 80% confluence. After removal of culture medium cells were washed with PBS, harvested, and nuclear extracts were prepared according to Dignam's method (Dignam et al., 1983). A Bio-Rad Protein assay was used to determine protein concentration.

The following oligonucleotides and their complementary sequences were used as probes in EMSA experiment: ARE (ARE site of human *GSTP1* promoter) - 5'-CGC CGT GAC TCA GCA CTG GG-3', NF-κB-like (NF-κB-like site of human *GSTP1* promoter) - 5'-TCC GCG GGA CCC TCC AGA AG-3', NF-κB (NF-κB site of human *GSTP1* promoter) - 5'-CTT AGG GAA TTT CCC CCC GC-3', CRE (CRE site of human *GSTP1* promoter) - 5'-GAG ACT ACG TCA TAA AAT AA-3', GATA (GATA-1 binding site of human *GSTP1* promoter) - 5'-GAG ATCA ATA TCT AGA AAT AA-3'. Probes were prepared by denaturation of complementary oligonucleotides for 2 min at 95 °C, annealing for 20 min at 60 °C and 30 min at 22 °C. Probes (10 pmoles) were incubated for 30 min with 20 pmoles [γ-P32]-ATP 6000Ci/mmol (Hartmann Analytic, Germany) in 20 µl of reaction mixture containing 10 u PNK (Roche, Switzerland) and PNK Buffer. Unincorporated nucleotides were removed by gel-filtration through Bio-gel P-30 (Bio-Rad, USA).

Five micrograms of cell nuclear extracts were preincubated for 10 min at 25 °C with 1 µg sonicated E. coli DNA and with or without 5-10 pmoles of unlabeled competitor in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 20% glycerol, 1 mM DTT. Then 0.1 pmole of [γ-P32]-ATP labeled probe was added to the mixture and incubated for a further 30 min at 25 °C. The reaction mixture was then loaded onto a prerun (200 V for 1 h at 4 °C) 6% native polyacrylamide gel (29:1 cross-linking ratio) containing 1x TBE. Electrophoresis was performed at 20 mA for 3h at 4 °C and the gel was then dried and radiographed. In supershift experiment the reaction mixture was preincubated for 20min at room temperature with 2 µg of antibody before the addition of radiolabeled probe. Consensus oligonucleotides for AP-1, NF-κB, CREB, GATA, ER and RAR, antibodies against human c-Jun (sc-44X crossreactive to JunB and JunD), c-Fos (sc-253X crossreactive to FosB and Fra2), MafF/G/K (sc-22831X), ERβ (sc-8974X), Nrf3 (sc-15460X), NF-κB1 p50 (sc-1191X),

NF-κBRelA/p65 (sc-7151) and normal rabbit IgG (sc-2027) were from Santa Cruz Biotechnology, USA.

3. Results

3.1 Me-45 melanoma cells reveal low level of *GSTP1* expression

At the first step of our study we examined *GSTP1* expression in Me45 cells in comparison with the other malignant cells including breast cancer cells MCF7, nonmalignant breast cells Hbl-100, bronchoalveolar carcinoma cells H358, alveolar carcinoma cells A549, myelogenous leukaemia cells K562, hepatocarcinoma cells HepG2, colon cancer cells Hct116, immortalized human bronchial epithelial cell BEAS2B, adrenal carcinoma cells FN-H296, choriocarcinoma cells BeWo and mammary carcinoma cells MCF10A. We estimated the amount of *GSTP1* mRNA in total RNA isolated from these cells (Fig. 2). The level of *GSTP1* mRNA in Me45 cells was significantly lower than in majority of other cells and slightly higher than in BeWo cells. The *GSTP1* mRNA content in MCF7 and HepG2 cells was below the level of detection.

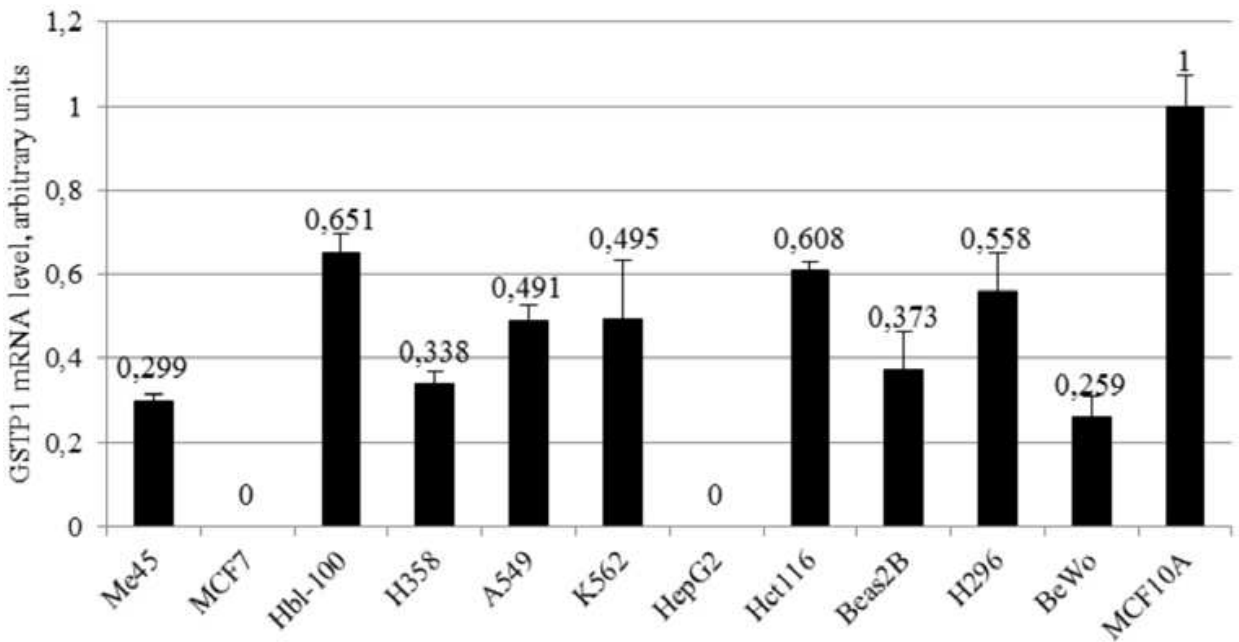


Fig. 2. Quantitative RT-PCR analysis of *GSTP1* mRNA content in malignant cells (Slonchak et al, 2009). The values reflect the ratios of the normalized *GSTP1* mRNA level in each cell type to the level in MCF10A cells that possess the highest *GSTP1* expression. The data were normalized to expression of RPL41 ribosomal protein gene and represented as the mean ± SD of three separate experiments with triplications in each

To verify whether the differences in *GSTP1* mRNA content are persistent at the protein level, the semi-quantitative Western-blot analysis was undertaken. The *GSTP1* protein content in Me45 cells was approximately 1.5-fold lower than in Hbl-100 cells and 1.9-fold higher than in BeWo cells (Fig. 3) that is compatible with the differences at mRNA level.

3.2 *GSTP1* expression in Me45 cells significantly elevates after γ-irradiation

The effect of ionizing radiation on *GSTP1* expression in Me45 melanoma cells was assessed by determination of *GSTP1* mRNA content in total cellular RNA before irradiation and at

0.25 h, 1 h, 3 h, 5 h, 8 h and 12 h thereafter (Fig. 4). It increased beginning from 0.25 h after irradiation and reached a nearly 5-fold increase at 8 h i.e. *GSTP1* expression in Me45 cells is up-regulated by γ -irradiation.

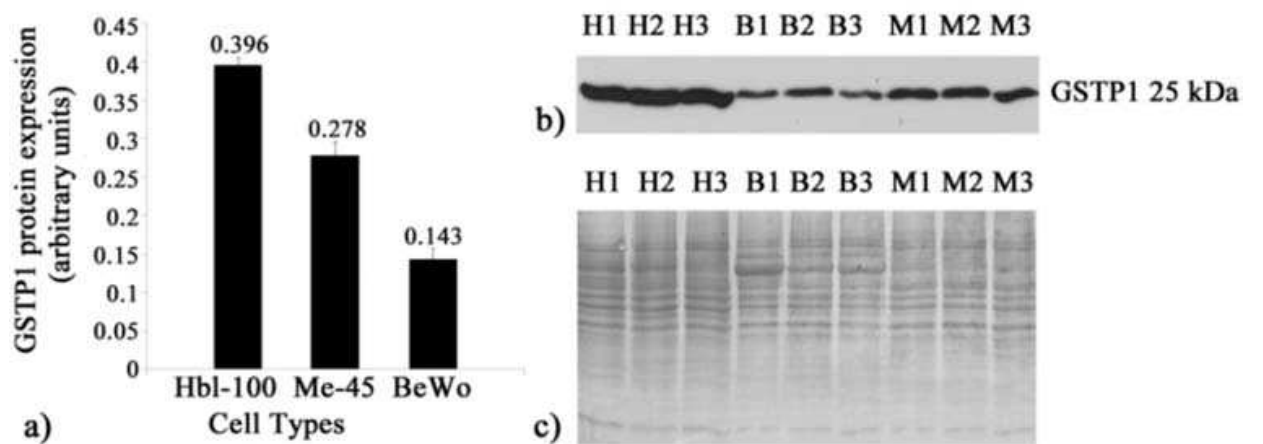


Fig. 3. *GSTP1* protein level in Hbl-100, Me45 and BeWo cells. a) The ratios of densitometry densities of *GSTP1* bands to the densities of the whole lane on Ponceau S-stained membrane are represented as the mean \pm SD of three separate experiments with triplications in each; b) The representative immunoblot probed with antibodies to *GSTP1*; c) The membrane stained with Ponceau S as a loading control. The letters H, B and M designate Hbl-100, BeWo and M45 cells correspondingly

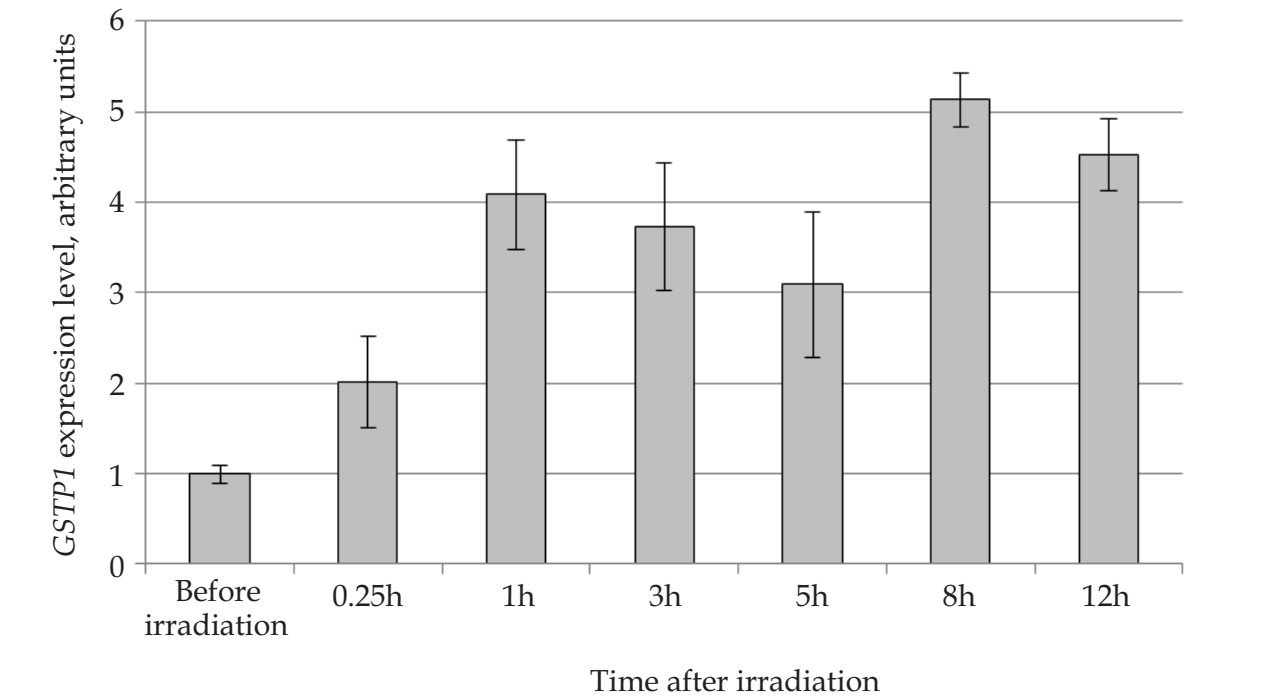


Fig. 4. Expression of *GSTP1* gene in Me45 cells before and after γ -irradiation. The values reflect the ratio of *GSTP1* RNA in each time point to the value before irradiation. The data were normalized to content of RPL41 ribosomal protein mRNA and represented as the mean \pm SD of three separate experiments with triplications in each

3.3 Regions of *GSTP1* promoter from – 1162 to – 405 and from – 105 to – 85 contain the negative regulatory elements and the region from – 405 to – 105 contains positive regulatory element in Me45 melanoma cells

The regulatory role of different regions of *GSTP1* promoter in Me45 cells was explored by transient transfection assay with the reporter constructs containing the firefly luciferase gene under the control of complete or truncated *GSTP1* promoter. We designed the reporter constructs each lacking the DNA fragment containing one transcription factor binding site. Transfection of the vector pGSTP1415 containing the complete *GSTP1* promoter (fragment from – 1379 to +35) resulted in relatively high level of *f-luc* gene expression (Fig. 5). Deletion of the flanking region from – 1379 to – 1162 with GATA-binding site did not change significantly the expression of the reporter gene. Further deletion of the region from – 1162 to – 405 with CRE resulted in increase of *f-luc* expression approximately 1.8-fold in comparison with previous construct. Deletion of the next region (from – 405 to – 105) containing NF-κB binding site, diminished expression of the reporter gene approximately 2.7-fold. Deletion of the region from – 105 to – 85, known as NF-κB-like element, heightened the *f-luc* expression 1.9-fold.

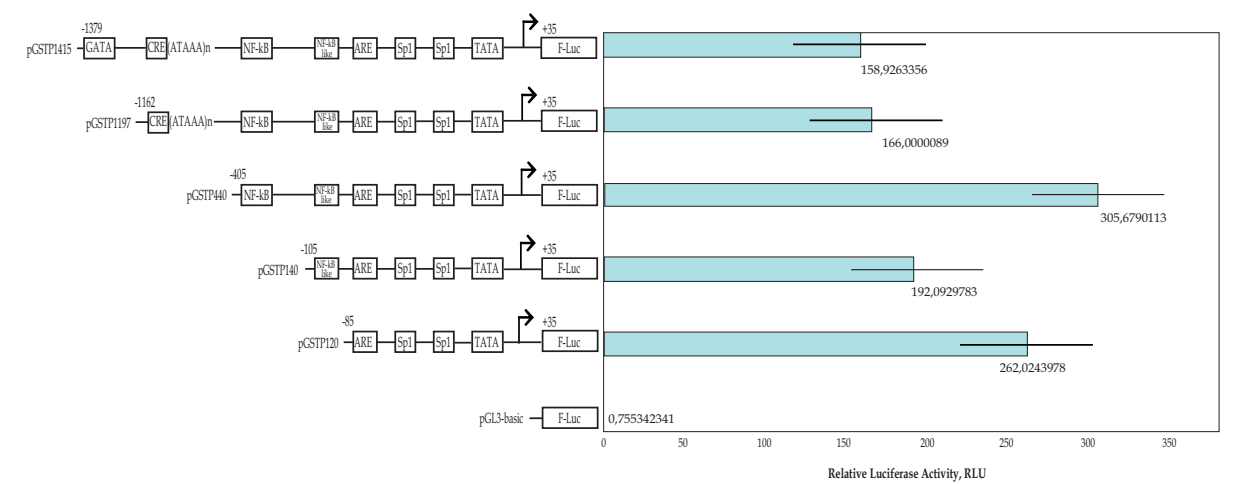


Fig. 5. Reporter constructs and relative luciferase activity in transfected Me45 cells. Data were normalized to expression of *renilla* luciferase from pRL-TK vector cotransfected together with the reporter constructs. Relative luciferase activity was calculated as a ratio of firefly to *renilla* luciferase light emission. Each bar in the figure represents the average ± SD of three independent transfection experiments with triplications in each

Thus transient transfection experiment revealed two negative regulatory elements located in the regions from – 1162 to – 405 and from – 105 to – 85 and the strong positive regulatory element located in the region from – 405 to – 105 in *GSTP1* promoter in Me45 cells.

3.4 Nuclear proteins from Me45 cells specifically interact with ARE, NF-κB-binding site and CRE, but not with NF-κB-like and GATA sites

To test the ability of the regulatory elements identified in *GSTP1* promoter to interact with nuclear proteins from Me45 cells the electrophoretic mobility shift assay (EMSA) was performed. The ability of 20 bp promoter fragments, containing ARE, NF-κB-like, NF-κB, CRE and GATA sites to bind nuclear proteins from Me45 cells was examined in this experiment. Fig. 6 indicates that all oligonucleotides form complexes with Me45 nuclear proteins.

The specificity of protein binding was examined in a competition experiment, in which nuclear proteins were preincubated with 50- and 100-fold molar excess of unlabeled probe. This experiment demonstrated that CRE, NF- κ B and ARE, but not NF- κ B-like and GATA sites specifically bind nuclear proteins (Fig. 6B, C, Fig. 7). The complexes marked as non-specific were not eliminated by excess of any unlabeled oligonucleotide identical to that used as a probe.

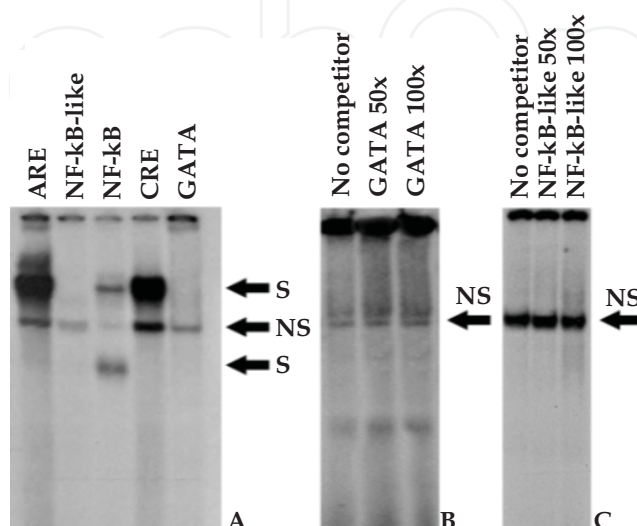


Fig. 6. In vitro binding of Me45 nuclear proteins to the *GSTP1* promoter sites. Electrophoretic mobility shift assay demonstrates that Me45 nuclear proteins form complexes with ARE, NF- κ B, NF- κ B-like, CRE and GATA sites. Results of competitive EMSA demonstrate that protein binding to NF- κ B-like and GATA site is nonspecific. S - specific complex, NS - non-specific complex

3.5 Transcription factor NF- κ B, and ER β in complex with Fos or unidentified protein interact with *GSTP1* promoter elements in Me-45 cells

To identify the transcription factors that interact with *GSTP1* promoter in Me45 cells we used a competitive EMSA with the oligonucleotides of two types - those containing consensus cis-elements to the potential transcription factors and authentic cis-elements (fig. 7).

For identification of transcription factors that bind ARE site, a 50- and 100-fold molar excess of unlabeled oligonucleotides containing consensus binding sites for transcription factor AP-1, DNA-binding protein Maf (which mediates interaction of NF-E2 and related factors with DNA), estrogen receptor β (ER β) and retinoic acid receptor (RAR) were used. Neither of these consensus oligonucleotides competed for transcription factors with authentic ARE site (Fig. 7a). It means that DNA-binding domains of transcription factors that recognize consensus oligos do not interact with ARE site. This results were verified by supershift experiments with polyclonal antibodies to c-Jun (cross-reactive to JunB and JunD), c-Fos (cross-reactive to FosB, Fra1 and Fra2), MafF/G/K, ER β and Nrf3. As indicated in Fig. 7b, neither Jun, Fos nor Maf and Nrf3 transcription factors are involved in formation of specific complex with ARE site. However, antibody to ER β disrupted the original complex and the new complex with higher electrophoretic mobility was formed. Therefore it is plausible that ER β binds to the *GSTP1* ARE site via another yet unidentified protein but not with its DNA-binding domain.

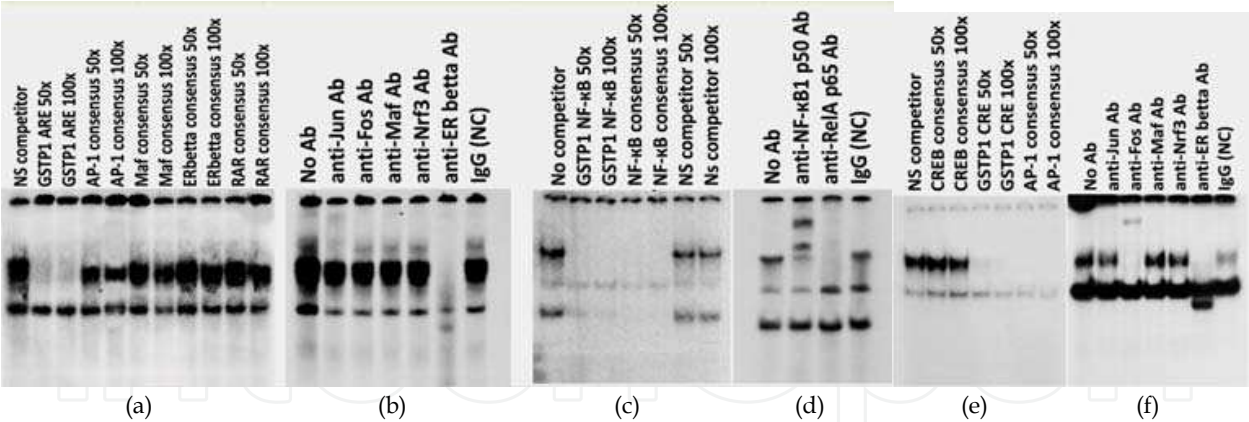


Fig. 7. Analysis of the complexes formed by ARE, NF-κB and CRE sites of *GSTP1* promoter with nuclear proteins. Formation of ARE-protein complex is inhibited by unlabeled authentic oligo with ARE site (a) and by ERβ antibody (b); NF-κB-site forms two complexes, both disrupted by the authentic corresponding oligo and oligo with NF-κB consensus (c); p50 antibody supershifts both complexes and p65 antibody disrupts the upper complex (d); The complex of CRE site with nuclear proteins is inhibited by authentic oligo, oligos with AP-1 consensus, but not CRE one compete with the CRE containing oligo (e); antibodies to Fos and ERβ supershift the complex (f)

The results of EMSA with oligonucleotide containing NF-κB site of *GSTP1* are represented in Fig. 7c. Two specific complexes were detected in the reaction with nuclear proteins. The unlabeled oligo with NF-κB consensus efficiently competed with the authentic oligo containing NF-κB response element for the nuclear proteins involved in formation of upper and lower complexes (Fig. 7c).

The composition of two different complexes with NF-κB response element was identified by supershift experiment with polyclonal antibodies to NF-κB1 p50 and RelA p65 subunits. Preincubation of nuclear proteins with antibody to p50 resulted in appearance of two supershifted bands (Fig. 7d). It means that both initial complexes contain p50 subunit of NF-κB. In the presence of p65 antibody the upper band disappeared pointing to the involvement not only p50 but also p65 subunit in this complex. Hence we provided the evidence that p50/p50 homodimer and p50/p65 heterodimer interact with *GSTP1* NF-κB binding site in Me45 cells.

To test the ability of CRE site to interact with CREB and AP-1 proteins, the corresponding oligonucleotides containing consensus binding sites for both transcription factors were used in the competition experiments. An autoradiograph in Fig. 7e indicates that oligo with consensus sequence to CREB did not compete with authentic oligo, while cold authentic probe and oligo with consensus to AP-1 competed successfully. Therefore it means that transcription factor AP-1 binds to CRE in Me45 cells.

The composition of CRE-protein complex was investigated by the supershift experiment with to Jun, Fos, MafF/G/K, ERβ and Nrf3. Incubation with antibodies to Fos and ERβ resulted in appearance of the new bands (Fig. 7f). Therefore both transcription factors take part in the complex formation with CRE in melanoma Me45 cells.

Thus, we demonstrated that positive regulatory element ARE interacts with ERβ in complex with another protein, negative regulatory element CRE interacts with Fos/ERβ-complex and positive regulatory element NF-κB binding site interacts with transcription factor NF-κB in the form of p50/p50 homodimer and p50/p65 heterodimer.

3.6 γ -Irradiation induces *GSTP1* expression in melanoma cells Me45, but not in choriocarcinoma cells BeWo

Transcription factor NF- κ B (p65) is known to be activated during oxidative stress particularly that created by γ -irradiation and hydrogen peroxide (Gilmore, 1999). Taking this fact into account we have supposed that p50/p65-heterodimer of NF- κ B, identified as a part of DNA-protein complex formed on *GSTP1* promoter in Me45 melanoma cells, may be responsible for *GSTP1* induction by γ -irradiation. To verify this assumption we used choriocarcinoma BeWo cells. The level of *GSTP1* expression in BeWo cells is very near to that in Me45 cells as it was shown in this study (Fig. 2). But as we have demonstrated previously the p50/p65-heterodimer is not involved in regulation of *GSTP1* transcription in BeWo cells while all other transcription factors are identical between two types of cells (Slonchak et al., 2009). As demonstrated in Fig. 8, *GSTP1* mRNA content in BeWo cells in contrast to Me45 cells does not change significantly after γ -irradiation. It is quite possible that the sole difference between transcription factors regulating *GSTP1* transcription in two types of cells, the presence/absence of p65 subunit of NF- κ B, mediates induction of *GSTP1* transcription in response to γ -irradiation in melanoma cells.

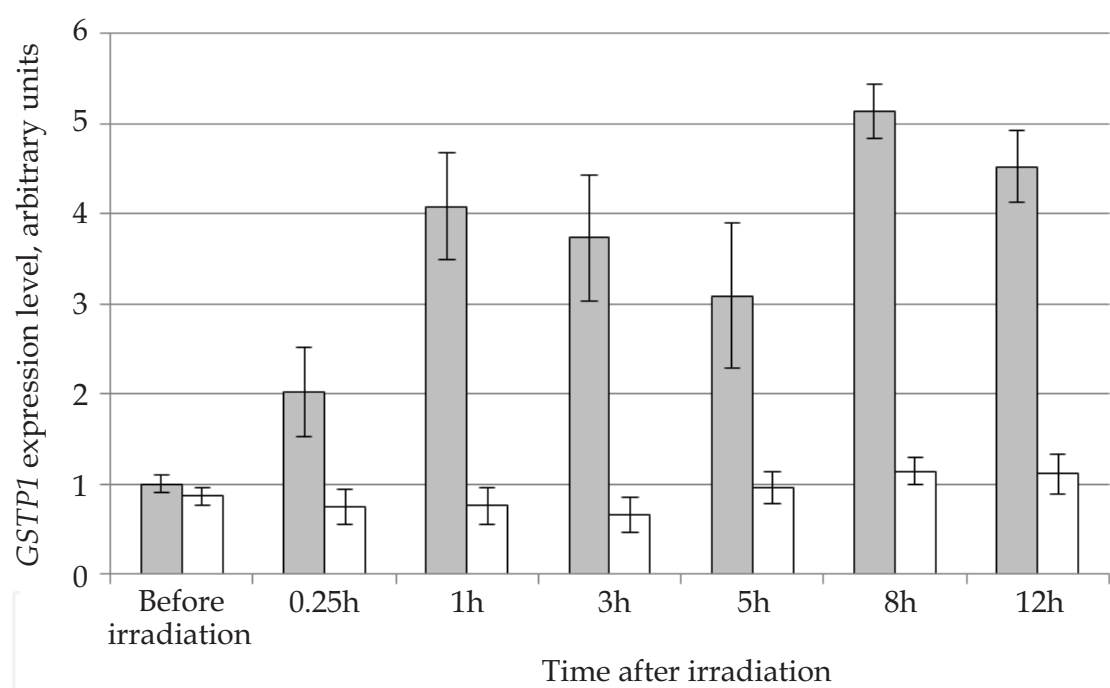


Fig. 8. Expression of *GSTP1* gene in Me45 and BeWo cells before and after γ -irradiation. Gray bars represent *GSTP1* mRNA level in Me45 cells and white bars – in BeWo cells. The values were normalized to expression of RPL41 ribosomal protein gene and presented as the ratios to *GSTP1* expression in both cells before irradiation. The graphs represent the mean data \pm SD of three separate experiments with triplications in each

4. Discussion

GSTP1 is a caretaker gene as it detoxifies endogeneous and exogeneous toxic compounds and thus protects the organism against genome damage, oxidative stress (Hayes & Strange, 1995), cancer (Henderson et al., 1998), and degenerative diseases (Guven et al., 2011). Down-regulation of *GSTP1* expression with concomitant decrease of detoxification is a common

precancerous event (Cookson et al., 1997; Meiers et al., 2007; Song et al., 2002) while its up-regulation at the later stages of tumor development causes multidrug resistance (Diah et al., 1999). The role of *GSTP1* in development of malignant melanoma and its response to traditional therapy is far from being clear.

Nowadays it is known that *GSTP1* is expressed in normal human melanocytes and its expression is retained after malignant transformation (Hanada et al., 1991). Malignant melanocytes reveal resistance to the components of traditional therapy e.g. to etoposide, cis-platinum, γ -irradiation, which provoke the transition of the cells into apoptosis. In comparison with some other malignant cells (human leukemia cells HL-60, rat rhabdomyosarcoma cells R1) melanoma cells Me45 reveal greater resistance to each of these components and less quantity of cells are susceptible to apoptosis (Kumala et al., 2003). To unravel the potential role of *GSTP1* in the malignant transformation of melanocytes and their resistance to traditional treatment we have focused our attention on the *GSTP1* expression in malignant melanoma cells and molecular mechanisms of its regulation at transcriptional level. For this aim we examined the level of *GSTP1* expression in malignant melanoma cells Me45 before and after γ -irradiation and characterized cis-elements of *GSTP1* promoter and transcription factors interacting with them.

Among 12 malignant cell lines *GSTP1* expression in Me45 cells was at the low level (the cells MCF7 and HepG2 do not express *GSTP1* due to complete methylation of *GSTP1* promoter and potent repression of gene transcription). The situation drastically changes after γ -irradiation. The Me45 and BeWo cells expressing *GSTP1* nearly at the same level before irradiation significantly differ from one another after irradiation. The *GSTP1* expression steadily rises in melanoma cells and remains at the same level in BeWo cells. It was known that *GSTP1* may inhibit pro-apoptotic signal transduction (Zhao et al., 2006; Zhou et al., 2005), conjugate etoposide to glutathione (Depeille et al., 2005) and by these ways protect the cells from apoptosis. Thereby the peculiarities of cell-specific regulation of *GSTP1* expression in melanocytes and its up-regulation after γ -irradiation may to some extent explain the development of melanoma and its extreme resistance to chemo- and radiotherapy.

Initiation of gene transcription in eukaryotes is a multistep process which includes chromatin remodeling, DNA demethylation, enhanaseosome formation and the assembly of the general transcription machinery. Each event in this process is strictly controlled by cell- and state-specific coordinated interactions between transcription factors, coactivators and corepressors with chromatin and transcription machinery components. In this study we focused on the functional role of cis-elements of *GSTP1* promoter and corresponding transcription factors in regulation of gene transcription in human malignant melanoma cells. The interaction of cis-elements of *GSTP1* promoter with transcription factors were examined in different malignant cells (Duvoix et al., 2003; Henderson et al., 1998; Jhaveri & Morrow, 1998). All these studies have shown that in different kind of cells cell-specific transcription factors bind the same response elements. So far the regulation of *GSTP1* transcription in malignant melanocytes was not addressed. For the first time we have assessed the functional activity of *GSTP1* cis-elements in malignant melanocytes and identified two elements - antioxidant response element (ARE) and NF- κ B binding site, that positively regulate *GSTP1* transcription, and cAMP response element (CRE), that regulates it negatively. The same was true for mammary epidermal cells Hbl-100 and choriocarcinoma BeWo cells (Slonchak et al., 2009). Therefore the different set of transcription factors may define either positive or negative regulation of *GSTP1* transcription in cell-specific manner.

In malignant melanocytes the positive regulatory element ARE interacts with ER β through another yet not identified protein. This fact distinguishes Me45 cells among other malignant

cells in which diverse transcription factors or in different way interact with this site. The diversity includes transcription factor AP-1 in breast cancer cells VCREMS (Moffat et al., 1994) and in leukemia cells K562 (Duvoix et al., 2004a); transcription factor NF-E2 in K562 cells (Borde-Chiche et al., 2001); RAR α in HeLa cells (Xia et al., 1996) and ER β in complex with Jun/Fos or with Nuclear Factor E2 related factor (Nrf2) in mammary carcinoma cells MDA-MB-231 (Montano et al., 2004). It is interesting that transcription factors Jun and Fos in the latter complex cannot activate *GSTP1* transcription themselves but they recruit ER β and the whole complex substantially activates transcription and mediates up-regulation of *GSTP1* expression by estradiol and antiestrogen tamoxifen (Montano et al., 2004).

It is also noteworthy that in Me45 cells ER β indirectly interacts not only with antioxidant response element ARE, but also with cAMP response element CRE in complex with transcription factor Fos. In *GSTP1* promoter CRE was first identified in lung carcinoma cells Calu-6. In these cells it interacts with the transcription factor CREB-1 and mediates induction of *GSTP1* transcription by cAMP (Lo & li-Osman, 2002). In contrast to this communication we demonstrated that in Me-45 cells CRE acts as a negative regulatory element. Thus in one type of cells CRE acts as a positive element interacting with CREB and in the other type of cells as a negative element directly interacting with Fos in complex with ER β . The similar phenomenon has been described for the gene encoding steroidogenic acute regulatory protein, which is up-regulated by CREB and down-regulated by Fos/Jun both interacting with CRE site (Manna & Stocco, 2007).

The role of ER β in regulation of *GSTP1* expression was a disputable question until now. Possible implication of ER into regulation of *GSTP1* expression was demonstrated for the first time in 1988 by Moscow et al. who described the negative correlation between ER content and *GSTP1* expression in breast cancer cells (Moscow et al., 1988). Later Montano et al. demonstrated that ER β acts as a positive regulator of *GSTP1* expression in the same cells (Montano et al., 2004). Here we found that ER β indirectly interacts with two distinct sites of *GSTP1* promoter - positive regulatory element ARE and a negative regulatory element CRE and in both cases different transcription factors mediate the effect of ER β . We believe that these data may explain the previous contradictions - the ability of ER β to repress (Moscow et al., 1988) and induce (Montano et al., 2004) *GSTP1* transcription.

The region of *GSTP1* promoter from - 405 to - 105 which contains NF- κ B response element acts as a strong positive regulatory element in Me45 cells. It interacts with p50 NF- κ B1/p65 RelA heterodimer and with p50/p50 NF- κ B1 homodimer in Me45 cells and only with p50/p50 NF- κ B1 homodimers in BeWo cells (Slonchak et al., 2009). We suggest that activation of *GSTP1* transcription in Me45 cells by γ -irradiation and the insensitivity of BeWo cells to the influence of irradiation are connected with the differences in NF- κ B dimers in both types of cells. Proteins from NF- κ B family may be subdivided into two classes according to the sequence of their C-terminal domain, presence of transcription activation domain (TAD) and the sequence specificity of response elements with which they interact. Members of the class I (NF- κ B1 p50 and NF- κ B2 p52) do not contain TAD and have long C terminal domains with multiple copies of ankyrin repeats that inhibit the ability of the factors to activate transcription. The second class of NF- κ B family includes c-Rel, v-Rel, RelB and RelA (p65). These proteins contain C-terminal activation domain TAD and can activate transcription (Schmid et al., 1994). The members of both classes in different combinations can form dimers with various regulatory potential. In different types of cells the homodimers p50/p50, p65/p65, c-Rel/c-Rel and heterodimers RelB/p50, RelB/p52, p65/c-Rel, RelB/p65 or RelB/c-Rel were identified (Neumann & Naumann, 2007).

The members of the first class bind preferably to GGGRN motif in promoters (R – purine base, N – any base). The members of the second class bind to YYCC motif (Y – pyrimidine base). Thereby p50/p50 homodimers bind to 11 bp sequence which contains two GGGRN repeats, separated by A/T-pair. The heterodimers consisting from the members of both classes bind GGRNWYYCC sequence (W – A or T) (Baldwin, Jr., 1996). The NF- κ B binding site of *GSTP1* promoter is GGGAATTTC sequence and it corresponds to typical binding site for NF- κ B heterodimers. The homodimers p50/p50 may also interact with this site but this binding is weak and does not result in transcription activation (Neumann & Naumann, 2007). In this regard only the heterodimers containing either RelA, RelB or c-Rel with p50 or p52 are able to activate *GSTP1* transcription. In this study we found that p50/p65 RelA NF- κ B heterodimer and p50/p50 homodimer interact with NF- κ B binding site of *GSTP1* gene in Me45 cells in comparison with p50/p50 homodimers interacting with this site in BeWo cells. The obtained results together with the above mentioned characteristics of the members of both classes of NF- κ B family explain very well the difference in response of Me45 and BeWo cells to γ -irradiation and the resistance of malignant melanoma cells to radiotherapy due to the activation of *GSTP1* expression. The important role of p50/p65 RelA NF- κ B heterodimer in this process is highly expected. We have to note that γ -irradiation causes degradation of NF- κ B inhibitor with which it is bound in the cytoplasm and translocation of NF- κ B into the nucleus with its subsequent participation in regulation of gene transcription.

5. Conclusions

Melanoma is a malignant tumor of melanocytes that occurs in result of collision of UV photon with a chromophore in a skin cell, production of reactive oxygen species and oxidative stress with the DNA damage. Conceptually, oxidative stress results from a change in the equilibrium between production of pro-oxidant and their consumption or deactivation, favoring an excess of pro-oxidant that have noxious consequences at the molecular and cellular levels. The cellular antioxidant defense relies to the great extent on a powerful sulfur redox chemistry response in which glutathione S-transferases are active players. Therefore a deficiency in the activity of GSTs may contribute to the risk of UV-induced melanoma.

It is notorious that melanoma is the most aggressive form of skin cancer, which is resistant to all current modalities of anticancer therapy, including chemo- and radiotherapy. The overexpression of GST and multidrug resistance protein 1 may confer to the resistance of melanoma cells.

We demonstrated that the level of *GSTP1* expression in malignant melanoma cells Me45 was significantly lower than in eight other malignant cell lines and similar to that in choriocarcinoma cells BeWo. Despite the similarity of *GSTP1* expression in nonirradiated Me45 and BeWo cells their response to γ -irradiation was different - the level of *GSTP1* steadily rises merely in Me45 cells, not in BeWo cells. To find out the clue for the difference in response of both types of cells to γ -irradiation we examined the regulation of *GSTP1* gene transcription. Two cis-elements, ARE and NF- κ B binding site, positively regulate *GSTP1* transcription, and element CRE regulates it negatively in Me45 cells. The same is true for BeWo cells as we have shown previously. In both types of cells cis-element ARE interacts with ER β through another yet not identified protein and cis-element CRE – with Fos/ER β dimer. However in Me45 cells NF- κ B binding site interacts with p50/p65 RelA and p50/p50 dimers of NF- κ B while in BeWo cells – only with p50/p50 dimer. The induction of NF- κ B in

response to γ -irradiation, the strong activating potential of p65 RelA subunit of NF- κ B and subsequent up-regulation of *GSTP1* expression may stipulate the melanoma cells resistance to radiotherapy. However this cause may not be unique.

According to our results γ -irradiation induces activation of *GSTP1* expression via transcription factor NF- κ B. Therefore we suggest that inactivation of NF- κ B may prevent melanoma resistance. Clinical trials with drugs that block NF- κ B are currently in progress with promising results. As reviewed by Lee and Burckart, the NF- κ B activation process can be inhibited by pharmacologic agents at each activation step: glucocorticoids inhibit NF- κ B by up regulating I κ B expression, cyclosporine and tacrolimus inhibit calcineurin, a phosphatase that indirectly induces I κ B degradation, deoxyspergualin inhibits nuclear translocation of NF- κ B, aspirin and salicylates inhibit I κ B phosphorylation, tepoxalin and antioxidants inhibit NF- κ B activation by influencing the redox state of the cell (Lee & Burckart, 1998). It's clear that development of specific inhibitory agents has to go in parallel with improvement of targeted drug delivery systems.

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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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University Campus STeP Ri
Slavka Krautzeka 83/A
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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